REMARKS

Claims 1 and 9, as previously amended September 10, 2003, are currently amended further herein.

Claim 9 has now been further amended, in accordance with the Examiner's suggestion to eliminate the recitation of "the original extract containing a broad range of extracted chemical compounds" from step (C). Support for this amendment is found, e.g., in original Claim 9.

Claim 1 has likewise now been further amended to eliminate the recitations of "the fluid extract containing a broad range of extracted chemical compounds" from steps (B) and (C). Support for this amendment is found, e.g., in original Claim 1.

The paragraph at page 3, lines 35-43 has now been replaced in order to correct an obvious typographical error in that the inadvertently omitted word "be" has now been added in the first sentence: "The organism from which the biological material is obtained may **be** any . . . organism . . . ."

The three items raised in the September 26 and 29, 2003 telephone communications are addressed below.

Item 1. Claim 9, and Claims 10-16 depending therefrom, stand rejected under 35 U.S.C. §112, ¶2, for alleged indefiniteness in the use of the relative term "broad" in the Claim 9, step (C) recitation of "the original extract containing a *broad range* of extracted chemical compounds." Claim 9 has now been amended to delete this express recitation.

Applicants believe that Claim1, and Claims 2-8 and 18-21 depending therefrom, stand rejected for this same reason. As a result, Applicants have also now amended Claim 1 to delete, from steps (B) and (C), the express recitations of "the fluid extract containing a broad range of extracted chemical compounds."

Applicants point out that no subject matter has been surrendered by adding and/or removing the recitations of these "broad range" statements in Claims 1 and 9, as the aqueous isopropanol-based solvents recited in these claims has surprisingly been found capable of extracting an unexpected broad range of biochemical compounds from biological materials. (See further remarks regarding the breadth of extracted compounds provided under Item 3

below.) This property is thus present in the claimed subject matter by virtue of the selection of isopropanol as the alcohol used in the extraction solvents according to the present invention, with or without the express recitation. Applicants note that the September 10, 2003 amendment that added these recitations served as a clarification of an inventive feature of the claimed methods, in expressly reciting this feature already possessed by the invention as taught and claimed prior to the September 10, 2003 amendment. See, e.g., Specification page 2, lines 3-6.

Applicants believe that these amendments overcome the rejections and respectfully request that they be removed.

Item 2. Claims 1-8 and 18-21 stand rejected under 35 U.S.C. §112, ¶1, for alleged non-enablement for the full scope of "biological material." The rejection has been maintained for the stated reason that the Specification exemplifies the effectiveness of methods according to the present invention toward biological materials of only plants, fungi, and prokaryotes, but lacks exemplification in regard to biological materials of animals (including humans).

In the September 29, 2003 telephone conference, the Examiner explained that Applicants need to establish, either by argument or by submission of data, that methods according to the present invention are also effective toward biological materials of animals (including humans). Applicants provide the following remarks in order to establish this.

In regard to the range of biological materials for which the present invention is effective, the instant disclosure shows that the present invention is highly and reproducibly effective for metabolomic analysis of a variety of organisms and tissue and cell types. The representative Examples provided in the present Application demonstrate that the invention is effective to provide quantitative comparative analysis of metabolites from:

- Dicot plants (see Example 1, using leaf tissue cut from two growing tobacco cultivars);
- Monocot plants (see Example 2, using dry seeds from five corn varieties);
- Fungi (see Example 3, using lyophilized cultures of two different yeast species); and
- Prokaryotes (see Example 4, using lyophilized cultures of two bacterial species).

Thus, these Examples utilize leaves, seeds, and yeast and bacterial organisms, and dicot, monocot, fungal, and prokaryotic cell types.

Consequently, the disclosure exemplifies the practice of the inventive methods with some of the more difficult-to-extract cell types, as the exemplified biological materials represent a variety of walled cell types, including monocot, dicot, fungal, and bacterial walled cells. Although these cell walls vary in composition and structure, in each case they surrounding their cell's membrane(s). The chromatograms shown in the Figures associated with the Examples demonstrate the methods' ability to extract a wide range of cell metabolites from within each of these cell and tissue types, in particular from within the cytosols of the cells (i.e. intracellular metabolites). Thus, these Examples show that the inventive methods are effective in extracting intracellular metabolites from cells and cellular tissues in which a cell wall, i.e. an extra fibrous barrier, surrounds the cell membrane(s).

As a result, because the present methods have been clearly shown effective for walled-cell biological materials, their extension to other, non-cell-walled biological materials is not highly unpredictable. In the case of cells without walls, such as animal and human cells, and such as bacterial, plant, and fungal protoplasts, no such additional barrier of a fibrous wall need be overcome in order to extract intracellular metabolites. Therefore, Applicants believe that the scope of disclosure provided by the present Application bears a sufficiently strong correlation to the scope of the claims to enable the use of animal and plant cells and tissues, as well.

Also, the field of biochemistry has been well characterized in that it has established that all living things are carbon-based and rely on the same general classes of biochemicals, e.g.: carbohydrates and related alcohols and water-soluble acids, hydrocarbons and fatty acids and their derivatives, and amino acids and nitrogenous bases. All living things metabolize these classes of biochemicals by similar or identical chemical reaction types (condensation and hydrolysis reactions) and do so by means of many at least similar chemical reaction pathways. For example, essentially all living things utilize the glycolysis and citric acid cycles, wherein carbon is cycled through a number of water soluble organic acid metabolites including, e.g., malate, fumarate, citrate, isocitrate, ketoglutarate, oxaloacetate, and succinate. As a result, all living things can be found to contain a large proportion of similar or identical chemical species belonging to the same general classes of biochemicals, even though a significant fraction of individual chemical species may differ from one organism to another.

The present inventors have demonstrated that aqueous isopropanol-based solvents interact with these general classes of compounds in a manner that results in extraction of a very broad range of biochemical compounds belonging to a variety of dissimilar chemical classes, though in a manner that is generally reproducible among the variety of quite different cells and tissues tested. Because a very large proportion of the same or similar biochemicals occur in all living entities, the present invention can be readily practiced successfully with essentially any cell types, and because these biochemicals' interactions with the aqueous isopropanol-based solvents is quite consistent, the present disclosure involves technology that is not highly unpredictable.

By way of illustration, the chromatograms presented in the drawings show that of the dozens of species of indoles, sterols, and hydrocarbons shown as metabolite peaks for yeast extracts in Figures 11A-B, a large proportion of these metabolite peaks would appear identically for human and animal extracts, as well. The fatty acid metabolite peaks shown for yeast extracts in Figures 12A-B, which include the following, would also be largely identical for human and animal extracts, also: C12:0 (eluting at 8.67 min.), C14:0 (9.20), C16:0 (9.73), C16:1 (9.82), C18:0 (10.38), C18:1 (10.53), C20 (10.95), C22 (11.32), and C24 (at 11.94 min.). Likewise, a large proportion of the sugar, sugar acid, and water soluble organic acid metabolite peaks shown for yeast in Fig. 15D, including those eluting at 5-8 minutes (e.g., water soluble acids including malate, fumarate, citrate, isocitrate, ketoglutarate, lactate, oxaloacetate, pyruvate, and succinate) and those eluting at 10-13 minutes (e.g., sugars including glucose, fructose, mannose, and maltose) would be identically present for human and animal extracts.

Thus, a large number of the metabolites present in human and animal cells and tissues are identical to those of yeast (and other) species exemplified, and the majority of metabolites common to both types of cells fall largely within the same general types of chemical classes, which interact with the extraction solvents of the present invention in a reasonably predictable manner. Therefore, because the disclosure sufficiently teaches one of ordinary skill in the art how to successfully practice the present invention to extract and analyze a broad range of metabolites from plant, yeast, prokaryotic, and animal and human biological materials, Applicants respectfully submit that the scope of the disclosure provided by the instant Application bears a sufficient correlation to the scope of the claims to enable the use of any "biological material."

Applicants also note that the legal issue of the unpredictability of biological activities and properties of chemical/pharmaceutical and biotechnology inventions, which has been noted as an important factor in the legal analysis of enablement for such inventions, does not apply in parallel to the present invention. See, e.g., MPEP §2164.03 and MPEP §2164.08 (Rev. 1, Feb. 2000), and cases cited therein. As noted above, instead of relying on unpredictable biological activities and properties, the methods of the present invention rely on reasonably predictable interactions between chemical and biochemical molecules. Thus, for this reason, too, Applicants respectfully submit that the scope of the disclosure provided by the instant Application bears a sufficient correlation to the scope of the claims to enable the use of any "biological material."

Applicants believe that these remarks overcome the rejection and respectfully request that it be removed.

Item 3. Claims 1-8 and 18-21 stand rejected under 35 U.S.C. §103(a) for alleged obviousness over Chappell *et al.* (US 5,589,619) and/or over Chappell *et al.* in light of Waggle *et al.* (US 6,544,566). The rejection has been maintained for the stated reason that the unexpected results obtained by Applicants' selection of aqueous isopropanol-based extraction solvents have not been sufficiently established over the teaching of Chappell *et al.* that a variety of organic solvents -- including methanol, ethanol, and isopropanol -- and water mixtures therewith are useful to extract metabolites from biological materials.

In the September 29, 2003 telephone conference, the Examiner explained that Applicants need to provide comparative data showing that selection of an alcohol other than isopropanol, i.e. methanol or ethanol, fails to achieve the unexpected results Applicants obtained by selection of isopropanol for use in an extraction solvent.

Applicants now enclose herewith an inventor's Declaration, under 37 CFR 1.132, describing and presenting the results of comparative tests of methanol and isopropanol performance in aqueous extraction solvents. These tests demonstrate that the performance of methanol was substantially inferior to that of isopropanol both in the numbers and amounts of chemical species extracted and in the range of chemical species extracted.

This Declaration also describes how, from the perspective of one of ordinary skill in the art, the present Application provides description and data showing the particularly broad

spectrum of biochemical compounds effectively extracted by use of the claimed aqueous isopropanol-based solvents.

This Declaration also describes additional surprising and unexpected advantageous features obtained by the selection of aqueous isopropanol-based extraction solvents, in contrast to the results obtained with other solvents. The Declaration further explains the limited teaching and focus of the cited references in describing the extraction methods they teach for use in obtaining only certain narrow types of compounds.

Nothing in the cited references suggests, in regard to the selection of aqueous isopropanol or aqueous isopropanol-KOH:

- that such an extraction solvent could obtain such a large number, quantity, and range of biochemical compounds in a single extraction step, in a reproducible manner, among such a wide variety of biological taxa, tissues, and cell types;
- that it could do so without undergoing solvent degradation or phase separation or otherwise contributing significant levels of impurities or other components that obscure visualization and quantitative comparison of the relatively low concentrations of metabolites available from biological materials; nor
- that it could result in extracts that allow fractionation, where it is used, to be performed so as to avoid significant multiple-partitioning of biochemical species and classes, and that permit the reproducible, substantially-mutually-exclusive fractionation of extracted metabolite classes that allows direct, chromatogram-comparison-based identification of meaningful metabolomic differences between samples tested in a fractionation-utilizing embodiment, without first having to perform any identification of chemical species for any chromatographic peak.

Applicants believe that, in this light, the present invention clearly exhibits surprising and unexpected advantages over the cited extraction-and-analysis methods. Applicants believe that these amendments overcome the rejections and respectfully request that they be removed.

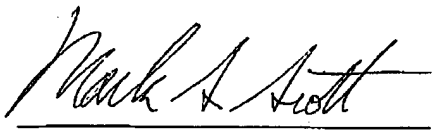
10/018,629

CONCLUSION

Applicants believe that the above remarks and amendments, in conjunction with the enclosed 1.132 Declaration, overcome the rejections maintained in the September 26 and 29, 2003 telephone communications. Applicants respectfully request that these rejections be removed in light of these remarks, amendments, and Declaration, together with the teachings of the present Application.

Applicants request that if there are any issues remaining unresolved regarding any points raised by this Action or that if no Claims are found allowable after consideration of this Supplemental Response, that an interview by telephone or in person be granted, to be arranged for a mutually convenient time. Reconsideration and allowance of the Application are requested in view of the above.

Respectfully submitted,



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Enclosure

- 37 CFR 1.132 Declaration of Inventor Nile N. Frawley (9 pages).

D. Lawrence
#6
10.15.03

IN THE UNITED STATES PATENT AND TRADMARK OFFICE

Applicants: Curtis D. Pfeiffer, Nile N. Frawley, et al.

Serial No.: 10/018,629 Group Art Unit: 1743

Filed: December 14, 2001 Examiner: Jan M. Ludlow

For: METHOD FOR CHEMICAL ANALYSIS OF BIOLOGICAL MATERIAL

CERTIFICATION OF FACSIMILE TRANSMISSION

I hereby certify that this paper is being facsimile transmitted to the U.S. Patent & Trademark Office to Examiner Jan M. Ludlow at: (703) 872-9310 on the date shown below:

October 10, 2003
DATE OF DEPOSIT

Melissa A. Wagner
PRINT OR TYPE NAME OF PERSON SIGNING CERTIFICATE

Melissa A. Wagner
SIGNATURE OF PERSON SIGNING CERTIFICATE

October 10, 2003
DATE OF SIGNATURE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION

STATE OF MICHIGAN)
) ss.
County of MIDLAND)

I, Nile N. Frawley, declare and state:

THAT I am an employee of The Dow Chemical Company (Dow);

THAT I am aware that the above-identified application is a national stage application, under 35 U.S.C. §371, of PCT/US00/19418, filed July 13, 2000, which claims the benefit, under 35 U.S.C. §119(e), of U.S. Serial No. 60/143,533, filed July 13, 1999;

THAT I am aware that the present application had an Official Action mailed June 10, 2003, which I have read along with the cited references, including that of Chappell *et al.* [U.S. Patent No. 5,598,619], that a Response thereto was made September 10, 2003, and that, pursuant to telephone communications with the Examiner on September 26 and 29, 2003, the

various obviousness rejections raised in regard to Claims 1-8 and 18-21 are being maintained until such time as evidence is submitted that substantiates that unexpected results were obtained by the selection of aqueous isopropanol-based extractants, in contrast to the performance of at least one other alcohol-based extractant among those listed at column 20, lines 20-27 of Chappell *et al.*;

THAT I am aware that Chappell *et al.*, at column 20, lines 20-27, list the following solvents as useful for extraction: methanol, ethanol, isopropanol, acetone, acetonitrile, tetrahydrofuran (THF), hexane, chloroform, combinations thereof, water mixtures therewith, vegetable oils, and steam distillation;

THAT, to overcome the rejections raised, the following data and remarks are submitted;

THAT, the inventors hereof considered other solvents, such as those listed by Chappell *et al.*, but excluded them for one or more of the following reasons, e.g.: that their extracts contained fewer species or lesser amounts of species than aqueous isopropanol; that they added impurities directly or by solvent degradation during processing and/or otherwise obscured the analysis, problems overcome by selection of aqueous isopropanol; and/or that, in embodiment in which fractionation is used, they did not permit substantially mutually exclusive partitioning during fractionation of chemical classes, which resulted in inability to readily quantitate a given species in a sample, a problem overcome by selection of aqueous isopropanol;

THAT, among the solvents listed by Chappell *et al.*:

(1) methanol was tried in direct comparison to isopropanol and found to be inferior as further described below;
(2) acetone, acetonitrile, and THF were tried and found to exhibit solvent instability in some embodiments and, in the case of THF, to add impurities at such high levels that observation of metabolites was obscured, making quantitation impractical;
(3) hexane and chloroform were tried and found to form two-phase extracts into which phases individual metabolites or metabolite classes differentially partitioned, ultimately making direct, chromatogram-based, comparative quantitative analysis impractical;
(4) vegetable oils were excluded because it is one object of the present invention to be able to identify quantitative differences in fatty acids and in waxes, terpenes, and other hydrocarbons, which metabolites are obscured by the fatty acids and other hydrocarbons of which vegetable oils consist, thereby making quantitation impractical; and
(5) steam distillation was excluded since it is an object of the present invention to maximize the variety of metabolites extracted in a single extract and steam distillation extracts only the limited range of steam-distillable volatile species;

THAT, in addition to the poorer performance exhibited by methanol in comparative tests, as further described below, the use of methanol to form the original extract also introduced a further problem, for embodiments in which fractionation is performed, in that it caused identical species and/or classes of compounds to partition into different fractions during fractionation of the original extract. This resulted in the further problem that, in the case of some metabolite compounds, the chromatogram generated for each fraction was not quantitative for a given metabolite or metabolite class. That, by way of example, I found that one or more fatty acids were variably, not reproducibly, partitioned into different fractions and thus appeared in different chromatograms; and that I believe one reason for this was that different subpopulations of fatty acids were spontaneously esterifying or de-esterifying at different rates;

THAT this multiple-partioning effect created the problem, in embodiments using fractionation, that direct subject-to-control chromatogram comparison did not provide reproducible, quantitative differences for each of the separated compounds. That further methods would, if possible, have had to be developed to *first* identify identical species in *different* chromatograms and then sum their peak values in order to subsequently permit quantitative comparisons against control values so as to ensure that identification of significant quantitative differences between subject and control could be obtained for each of the separated compounds. That, as a result, if methanol were selected in place of isopropanol, at least some chemical species identification would likely have to be performed up-front in the analysis in order to convert the chromatogram data into meaningful quantitative differences between subject and control;

THAT, in contrast to the methanol-based method, aqueous isopropanol-based methods of the present invention were unexpectedly found to permit the fatty acids, and other compound classes, to co-partition according to the chemical properties of those classes, where fractionation was used. That, unlike a methanol-based method, an aqueous isopropanol-based method of the present invention permits generation of chromatograms that are useful for direct comparison to identify quantitative differences, without having to first perform any chemical species identification(s). That, unlike a methanol-based method, an aqueous isopropanol-based method of the present invention now permits an analytical biochemist to first obtain a quantitative comparison of differences in species and classes by direct chromatogram comparison, only after which a species identification method would be needed, if any chemical species identification(s) are desired;

U.S. S/N 10/018,629

THAT the attached documentation of a comparative example that I have provided establishes the superior performance of aqueous isopropanol over that of aqueous methanol for use as an extraction solvent. In this representative comparative example, methanol (MeOH) was compared to isopropanol (IPA) in extracting metabolites from tobacco (*Nicotiana benthamiana*). The same pool of tobacco tissue was split into parallel samples. The first set of samples was extracted using 1:1 IPA:water (equal volumes) containing 0.1N KOH. The second set of samples was extracted using 1:1 MeOH:water (equal volumes) containing 0.1N KOH. Each of the resulting original extracts was then fractionated to produce Fraction 1 and Fraction 2. Fraction 1 is a first organic fraction, containing, e.g., pyridines, indoles, terpenes, phytols, sterols, and waxes; and Fraction 2 is a second organic fraction, containing fatty acids, derivatized as methyl esters. Representative chromatograms obtained for these Fractions 1 and 2 are presented as the four chromatograms shown on the attached sheet;

THAT, in this attached documentation, the Fraction 1 chromatograms for these IPA and MeOH extracts are respectively shown as the upper-left and lower-left chromatograms on the attached sheet. The MeOH Fraction 1 chromatogram presents fewer peaks and smaller peaks than the IPA Fraction 1 chromatogram. Overall, the MeOH peaks are only about 20% as large as the IPA peaks for this Fraction and, as seen by comparing the peaks falling within the the 11-14 minute elution range (the elution range for waxes), MeOH Fraction 1 presents many fewer wax species than IPA Fraction 1;

THAT, in this attached documentation, the Fraction 2 chromatograms for these IPA and MeOH extracts are respectively shown as the upper-right and lower-right chromatograms on the attached sheet. These two chromatograms present about the same number of peaks, but the MeOH Fraction 2 peaks are only about 50% as large as the IPA Fraction 2 peaks;

THAT I, either alone or in conjunction with inventor(s) listed on Patent Application No. 10/018,629, supervised and/or reviewed the performance of the tests for this comparative example, whose results are set forth on the attached sheet;

THAT these results exemplify that aqueous isopropanol performed unexpectedly well in comparison to aqueous methanol, with aqueous methanol performance being much less effective than aqueous isopropanol for extractions in regard to: (1) the number of chemical species extracted, (2) the amounts of chemical species extracted, and (3) the range of chemical species extracted, e.g., the highly non-polar metabolites (e.g., waxes);

THAT these and other tests demonstrate that, in comparison to other tested solvents, aqueous isopropanol was unexpectedly found able to extract a significantly broader range and/or number of chemical species in significantly larger quantities, without undergoing solvent

degradation or phase separation, without contributing significant levels of impurities or other components that obscure visualization and quantitative comparison of the relatively low concentrations of metabolites available from biological materials, and while unexpectedly permitting the reproducible, substantially-mutually-exclusive fractionation of extracted metabolite classes that allows direct, chromatogram-comparison-based identification of meaningful differences between samples, where fractionation is used;

THAT the cited references, including Chappell *et al.*, do not teach or suggest that isopropanol could provide any particular advantages over any of the other solvents listed therein, nor that any of the other listed solvents would exhibit drawbacks in metabolomic analysis;

THAT the above-captioned Patent Application provides description in the Specification and data in the Drawings that show that a particularly broad spectrum of biochemical compounds is effectively extracted by use of the claimed aqueous isopropanol-based solvents.

THAT the Specification, at page 11, line 1, to page 13, line 27, describes processes for fractionating the original extract obtained from the biological material, and this description indicates that a particularly broad spectrum of metabolites is effectively extracted by the methods of the present invention. This description indicates that, from the original extract:

- for example, pyridines, indoles, terpenes, phytols, alcohols (e.g., sterols), and hydrocarbons (e.g., waxes) are partitioned into Fraction 1;
- for example, fatty acids are partitioned into Fraction 2;
- for example, sugars and related compounds (among which I typically find, e.g., monosaccharides, disaccharides, sugar ethers, sugar acids and other water soluble acids, and also, e.g., inorganic phosphate), are partitioned into Fraction 3; and
- for example, amino acids are partitioned into Fraction 4.

Thus, this description explains that the use of aqueous isopropanol or aqueous isopropanol-KOH extraction solvents is effective to extract into the "original extract" at least biological pyridines, indoles, terpenes, phytols, alcohols, hydrocarbons, fatty acids, sugars and related compounds, amino acids, and inorganic compounds.

THAT, among the metabolites extracted in such representative extractions, I have regularly identified a very wide range of dissimilar chemical compounds. These compounds include those within the following classes:

- essentially lipophilic and non-polar compounds, which partition into Fraction 1;
- essentially amphiphilic compounds, which partition into Fraction 2;
- essentially hydrophilic and polar compounds, which partition into Fraction 3; and
- essentially amphoteric compounds, which partition into Fraction 4;

and I typically find that these extracted compounds fall both: within a broad molecular weight range of about 75, for glycolic acid, to just under 600, for some of the larger waxes; and within a very wide range of polarities including highly polar compounds, e.g., inorganic

phosphate having in excess of a -2 charge, and highly non-polar compounds, e.g., long-chain waxes;

THAT these four Fractions contain different sets of chemical compounds that have become partitioned, according to chemical classes based on their properties, into essentially mutually exclusive sets of compounds; and that this essentially mutually exclusive partitioning has been found reproducible between samples and between species tested, in embodiments in which fractionation is performed;

THAT I found it very surprising that the selection of aqueous isopropanol for use in forming the original extract was a key feature of the fractionation-using embodiments of the present invention that allowed this essentially mutually exclusive partitioning;

THAT this essentially mutually exclusive partitioning permits different chromatograms of the same type of fraction to be directly compared so as to allow immediate identification of quantitative differences between samples, without first having to first perform any identification of individual chemical species, in fractionation-utilizing embodiments;

THAT the chromatograms presented in the Drawings of the above-captioned Patent Application show that a particularly broad spectrum of metabolites is effectively extracted by the methods of the present invention. This is shown, e.g., by the multiple peaks present in the chromatograms for Fraction 1 (e.g., Figures 6A-6B, 8A-8E), Fraction 2 (e.g., Figures 7A-7B, 9A-9E), and Fraction 3 (e.g., Figures 7C-7D, 10A-10E, 15B-15E). The multiple peaks of each of these Fraction 1, 2, and 3 chromatograms represent a large number of individual species present in each Fraction. Thus, these chromatograms demonstrate that a broad range of chemically diverse species have been effectively extracted from the biological materials tested;

THAT a representative illustration of how the Drawings demonstrate this diversity of chemical species is seen in the results of Example 1. One of ordinary skill in the analytical biochemistry art, having read Example 1 at page 14, having read the page 11-13 description of fractionation procedure embodiments, and having read the page 13-14 description of the exemplified chromatography protocols, would recognize, for example:

- that the Figure 6A chromatogram presents in excess of 50 species of hydrocarbons and alcohols (e.g., sterols) partitioned into Fraction 1,
- that the Figure 7A chromatogram presents in excess of 30 species of fatty acids partitioned into Fraction 2, including C14:0, C16:0, C18:0, C18:1, C18:2, C20:0, C22:0, C24:0, and other fatty acids and isomers thereof, and

- that the Figure 7C chromatogram present in excess of 100 species of sugars and related compounds partitioned into Fraction 3, including monosaccharides, disaccharides, sugar ethers, sugar acids and other water soluble acids;

THAT, in regard to representative results for Fraction 4, I typically see in excess of 15 different amino acids in each Fraction 4 chromatogram;

THAT these representative chromatograms thus demonstrate that the selection of aqueous isopropanol-based extractants results in extraction of at least about 200 different chemical species in representative extractions;

THAT the cited references appear to indicate that extraction-and-analysis protocols can be selected or optimized to be reproducible for only a few chemical species at a time. For example, in the Chappell *et al.* patent, the extraction methods were selected or optimized for the extraction of squalene and sterols. Also, in the Waggle *et al.* patent, the extraction methods were selected or optimized for the separate extractions of:

- Sterols - obtained by steam-distilling volatiles away from expressed, crude soybean oil, followed by a molecular distillation of the resulting deodorized soybean oil (see col. 6, lines 36-64); and
- Isoflavone compounds and aglucones thereof - respectively obtained by extraction with "an alcohol," preferably methanol or ethanol (see col. 9 line 65); or with a "low molecular weight" alcohol (col 11, line 56);

each of which extractions is taught as optionally being followed by HPLC to achieve separation;

THAT the metabolites taught for extraction in the cited references represent only a very small subgroup of the compounds that the present invention effectively extracts. In the present invention, sterols, squalene, and aromatic alcohol metabolites, such as the isoflavone compounds, are a mere subpopulation of those extracted metabolites that become partitioned into Fraction 1 of a fractionation-utilizing embodiment of the present invention;

THAT, unlike the extraction methods taught in the cited references, the methods of the present invention do not need to be selected or optimized for any particular type or class of compound, but can be used as is for a very broad spectrum of different biological metabolite compounds belonging to a variety of different chemical classes, even though the present methods can optionally be modified, if desired, for particular types or classes of compounds;

THAT, it was very surprising to me that aqueous isopropanol, in comparison to the other tested solvents, was able to simultaneously provide the following combination of advantageous features: (1) extracting a significantly broader range and/or number of

chemical species in significantly larger quantities, (2) without undergoing solvent degradation or phase separation, (3) without contributing significant levels of impurities or other components that obscure visualization and quantitative comparison of the relatively low concentrations of metabolites available from biological materials; (4) allowing fractionation, where it is used, to be performed so as to avoid significant mutliple-partitioning of biochemical species and classes; and thus (5) permitting the reproducible, substantially-mutually-exclusive fractionation of extracted metabolite classes that allows direct, chromatogram-comparison-based identification of meaningful metabolomic differences between samples that are tested in a fractionation-utilizing embodiment.

THAT the lead inventor of the key cited reference, Dr. Chappell, expressed to me his own surprise at the advantages offered by the method of the present invention, during confidential discussions between inventors and Dr. Chappell, in September 1999.

The undersigned DECLARANT declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Nile N. Frawley

Nile N. Frawley

Date: 10/10/2003

Enclosure: Attachment Sheet of 4 Chromatograms (1 page)